7-16-2019

Biophysical characterization approaches to aid the selection of protein formulations by predicting their physical stability during long-term storage

Hristo Svilenov

Gerhard Winter

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Hristo Svilenov, Gerhard Winter

Department of Pharmacy

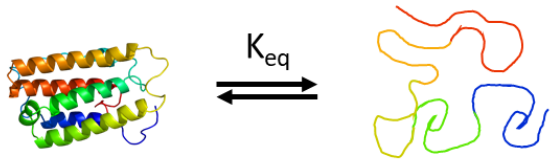
Ludwig-Maximilians-Universität München

July 2019

Protein stability aspects

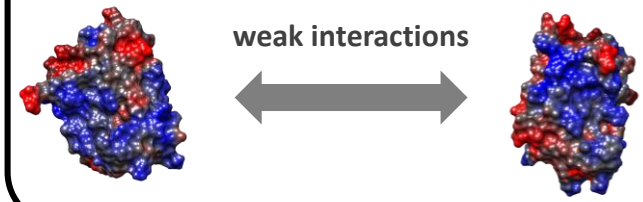
Physical stability

Conformational stability

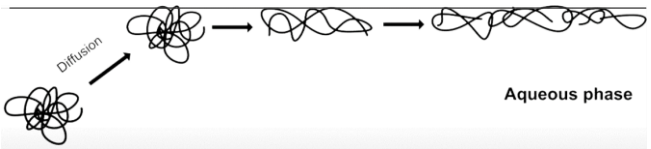


Folded protein — Unfolded protein

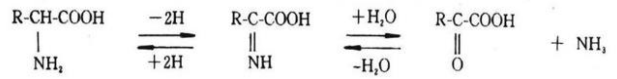
Colloidal stability



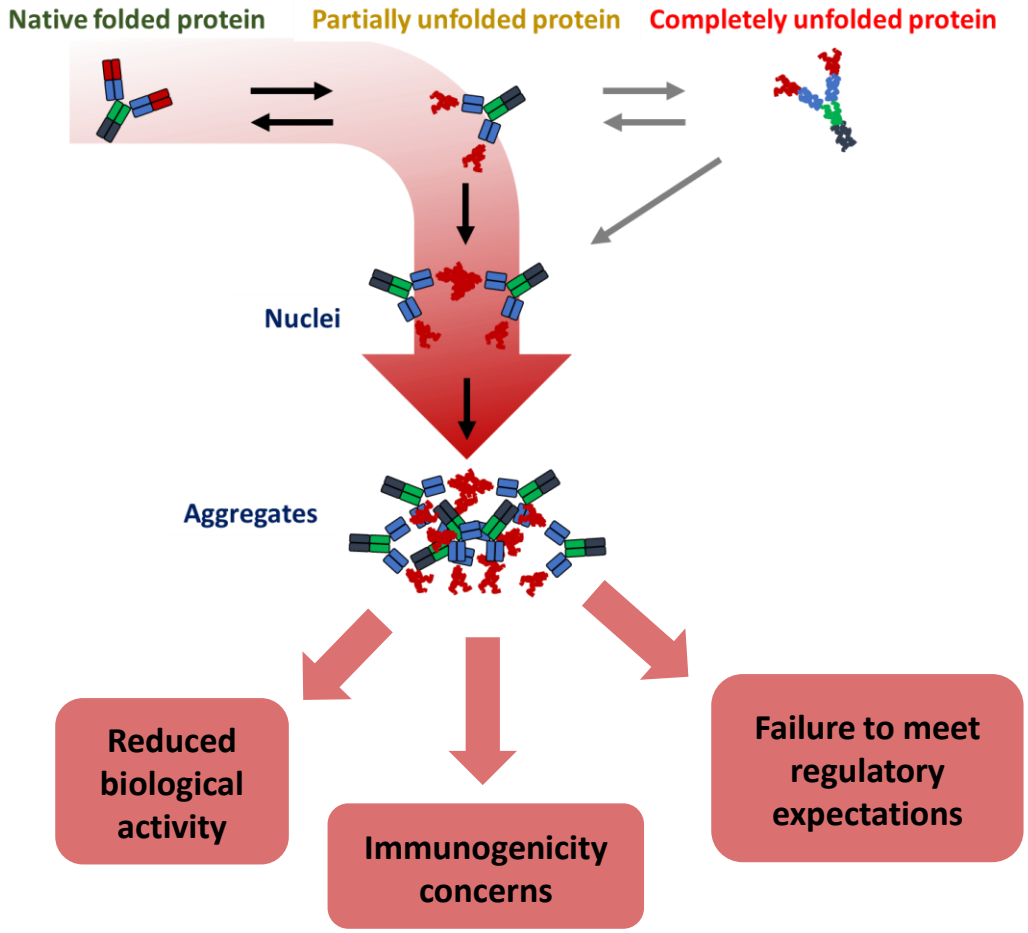
Interfacial stability



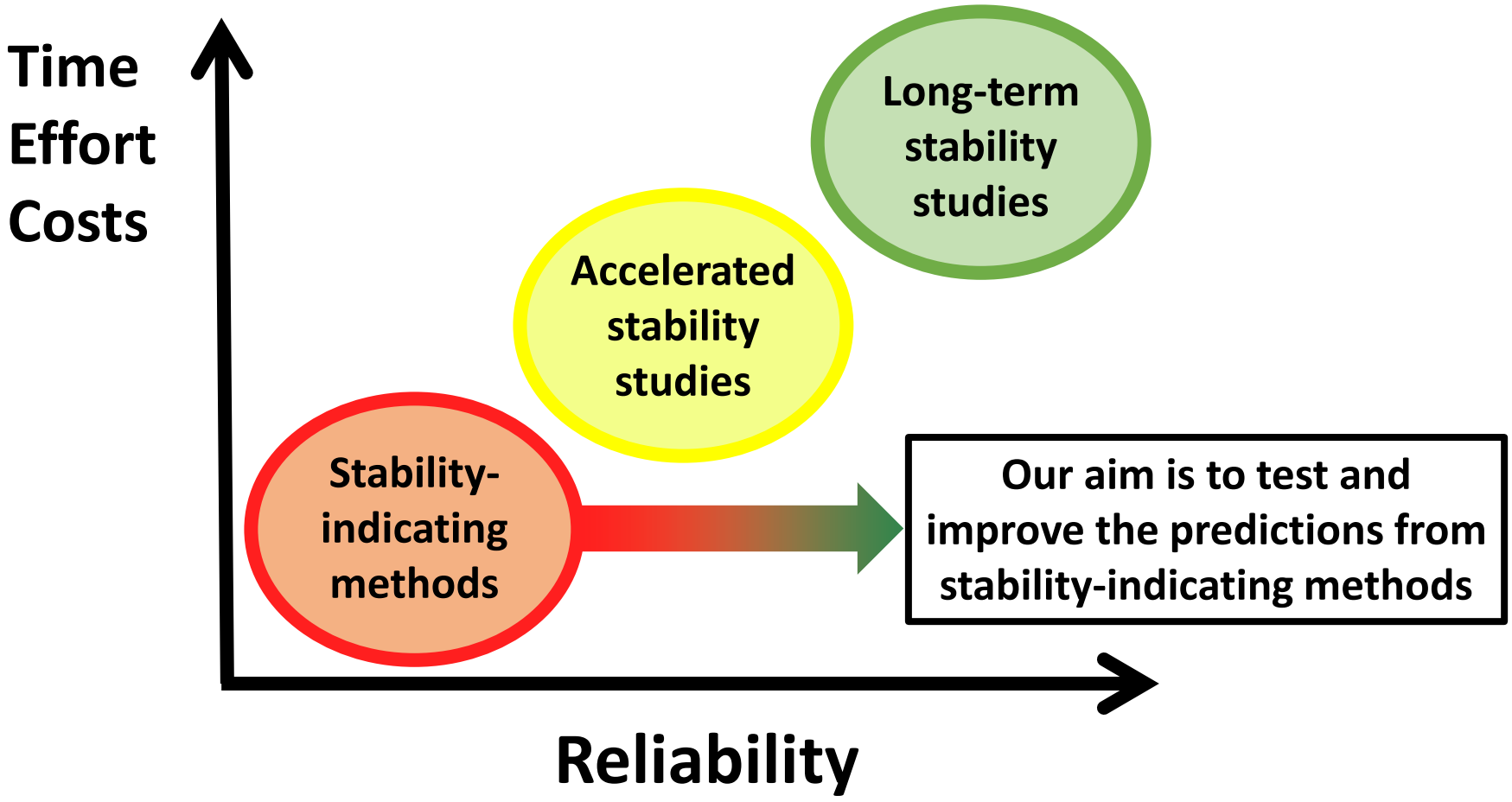
Chemical stability

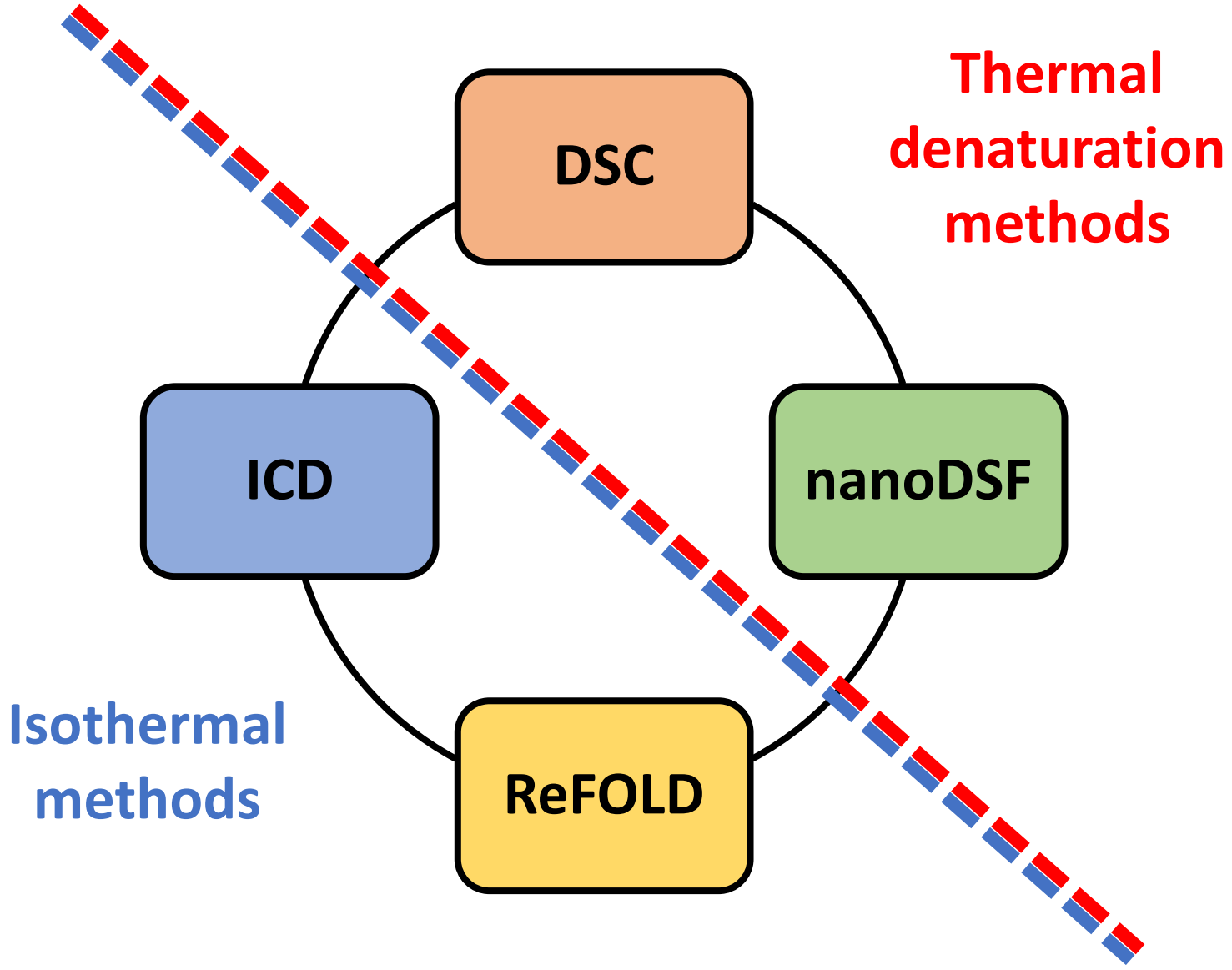


Protein aggregation due to poor physical stability

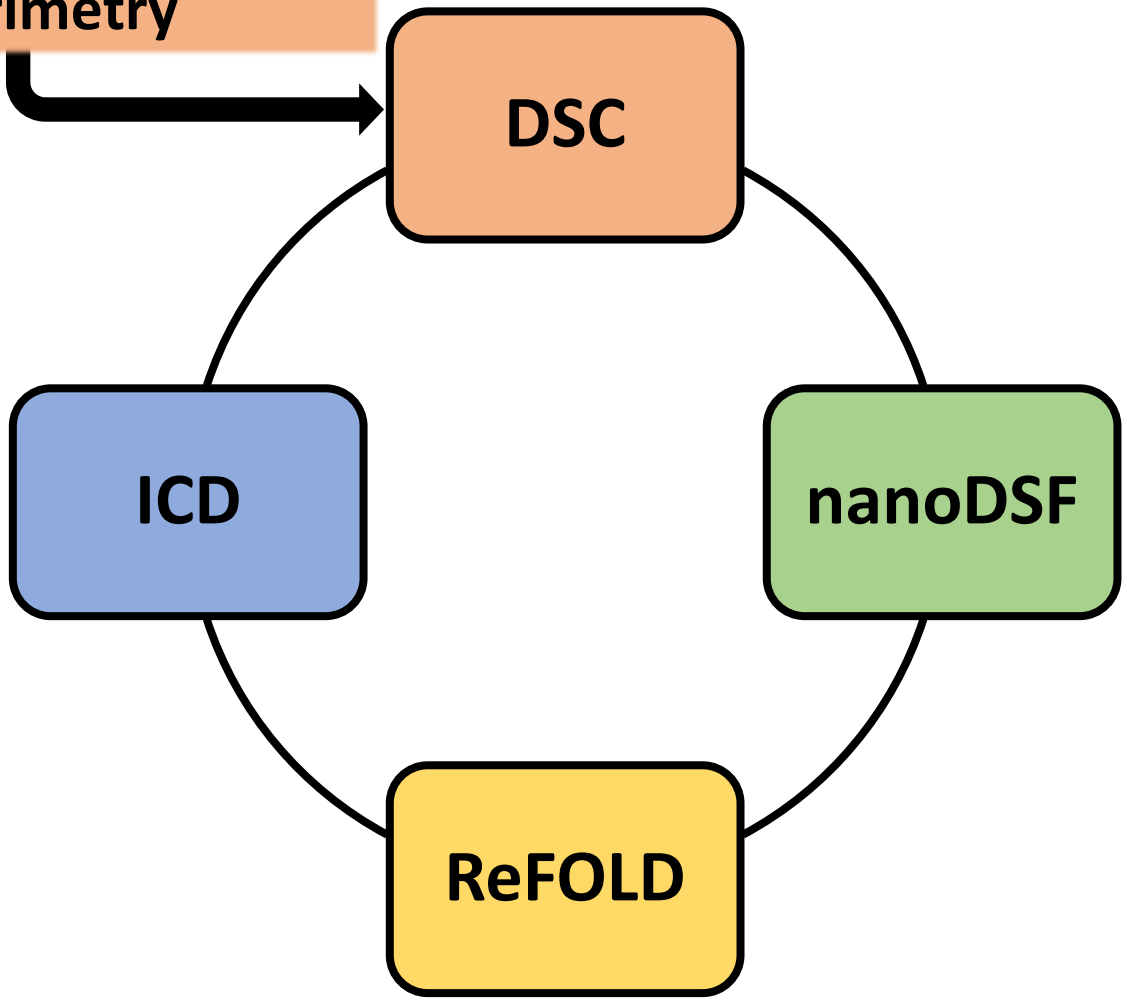


How to select formulations where protein aggregation is suppressed during storage?

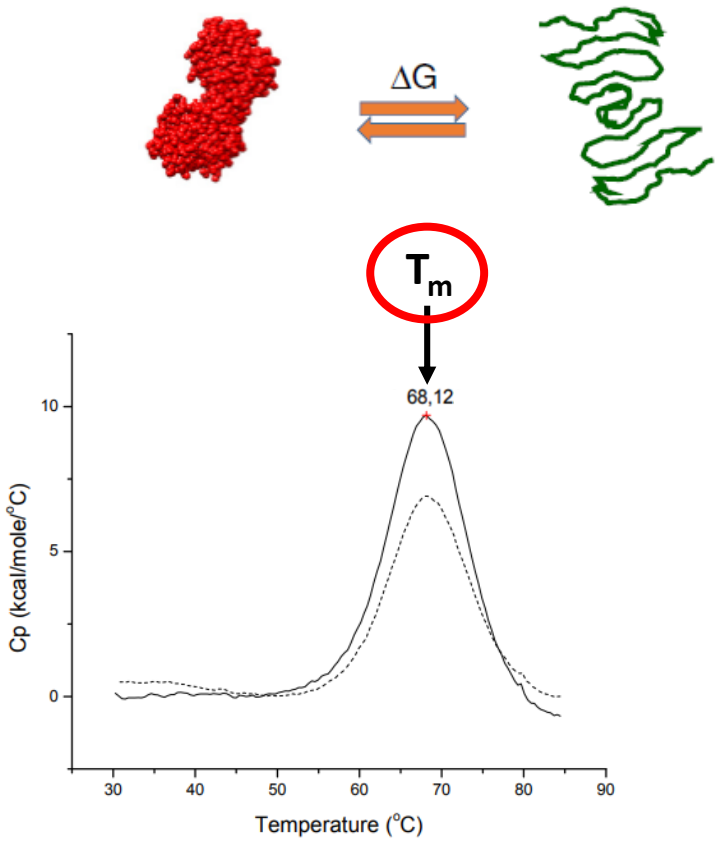


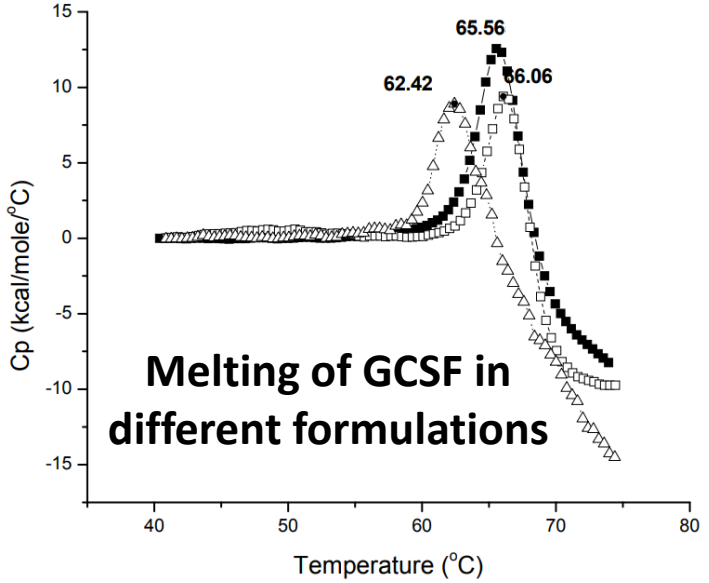

DSC
ICD
nanoDSF
ReFOLD
Thermal denaturation methods
Isothermal methods

Differential Scanning Calorimetry



Differential scanning microcalorimetry can be used to determine the (apparent) protein melting temperature T_m in different formulations

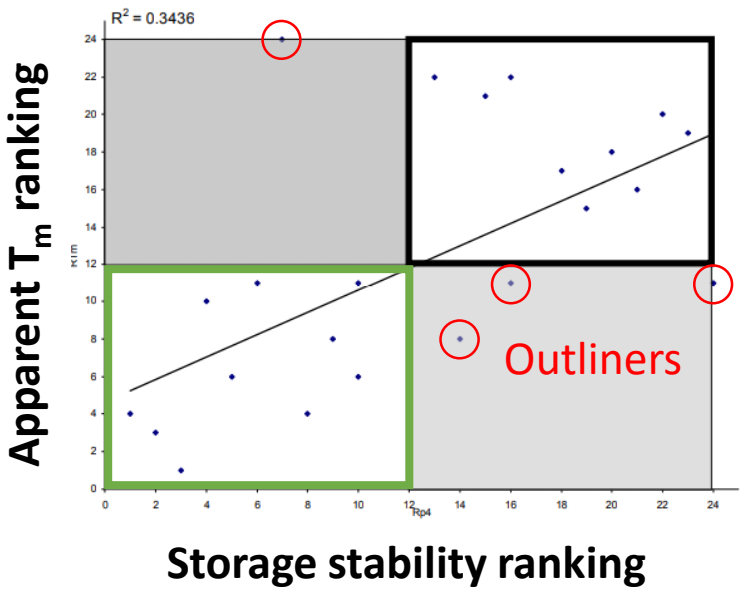




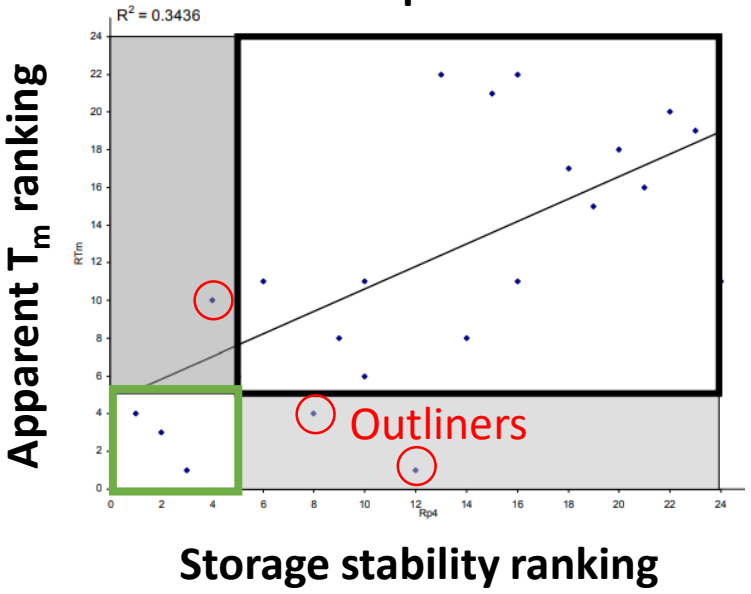
Basic assumption of this approach:
The long-term stability of the formulations correlates with the (apparent) protein melting temperatures

Correlation between prediction from melting temperature of GCSF and storage stability at 4 °C (Youssef and Winter 2013)

Best 50 % prediction



Best 20 % prediction



Other examples showing that DSC can provide only some predictions for storage stability:

Interleukin-1 Receptor (IL-1R) Liquid Formulation Development Using Differential Scanning Calorimetry

Richard L. Remmele, Jr.,[1,3] Nancy S. Nightlinger,[1] Subhashini Srinivasan,[2] and Wayne R. Gombotz[1]

molecular pharmaceutics
Article
pubs.acs.org/molecularpharmaceutics

Examination of Thermal Unfolding and Aggregation Profiles of a Series of Developable Therapeutic Monoclonal Antibodies

Mark L. Brader,* Tia Estey, Shujun Bai,† Roy W. Alston, Karin K. Lucas,‡ Steven Lantz, Pavel Landsman, and Kevin M. Maloney

MABS
2016, VOL. 8, NO. 6, 1088–1097
http://dx.doi.org/10.1080/19420862.2016.1189048
Taylor & Francis
REPORT

A comparison of biophysical characterization techniques in predicting monoclonal antibody stability

Geetha Thiagarajan[a], Andrew Semple[a], Jose K. James[b], Jason K. Cheung[a], and Mohammed Shameem[a]

[a]Sterile Product and Analytical Development Group, Biologics & Vaccines, Merck & Co., Inc., Kenilworth, NJ, USA; [b]Center for Advanced Biotechnology and Medicine, Robert Wood Johnson Medical School, Rutgers University, Piscataway, NJ, USA

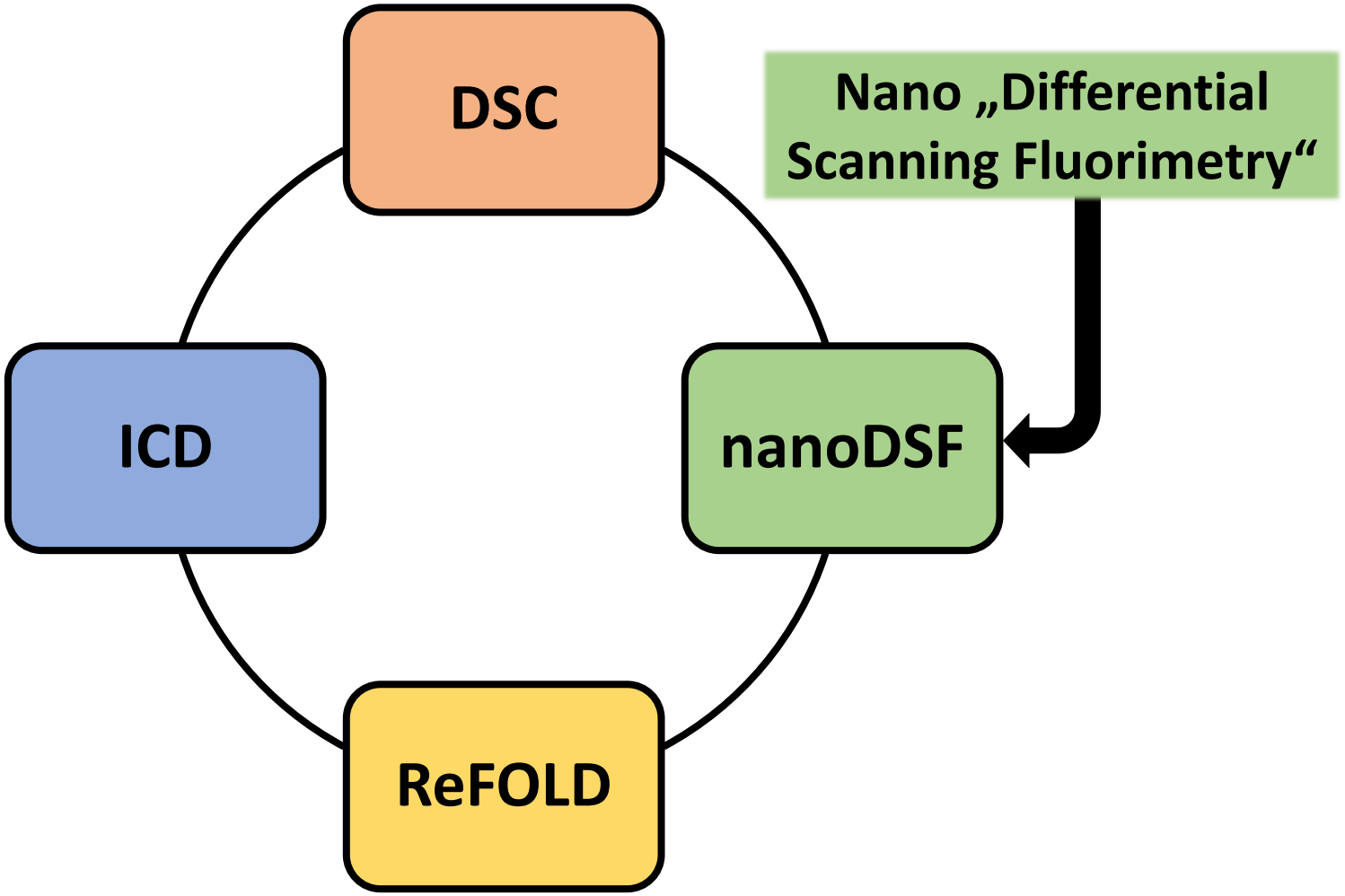
The T_m approach with DSC in brief

Measuring T_m with differential scanning calorimetry (DSC) offers:

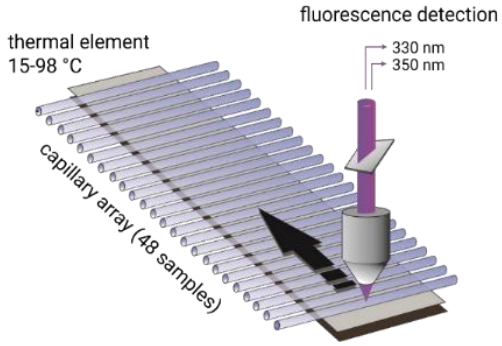
- Some predictions which formulations are stable during storage

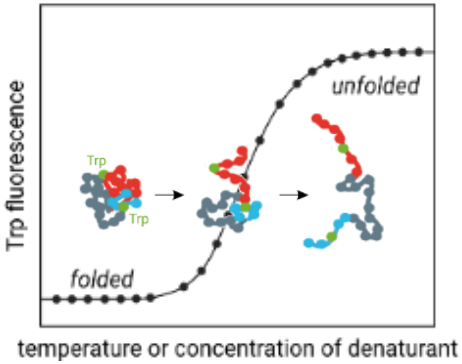
However, this approach had the following limitations:

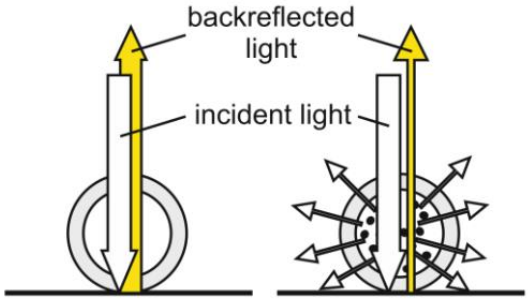
- Low-throughput: only one sample per run possible (ca. 3 samples per day)
- Sample heating: some excipients change properties with temperature
- Large sample volume: 550 µl measurement volume -> 800 µl handling volume
- Limited prediction reliability: "T_m showed a limitation in predicting the exact ranking"


DSC
Nano „Differential Scanning Fluorimetry“
ICD
nanoDSF
ReFOLD

nanoDSF measures protein unfolding by detecting changes in the intrinsic protein fluorescence

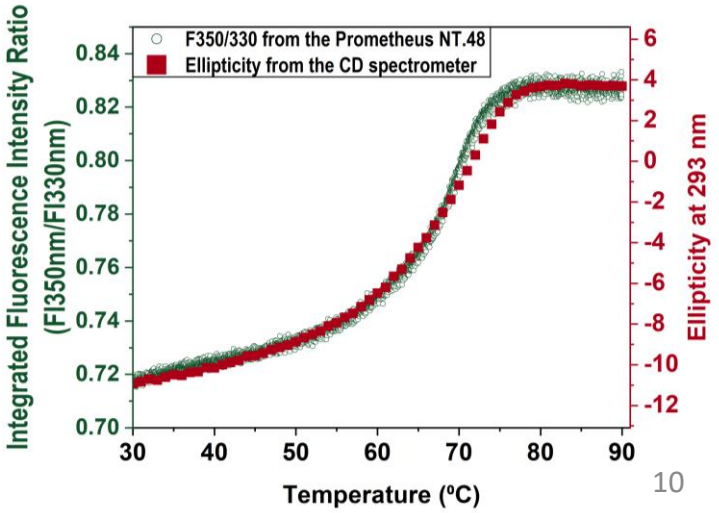




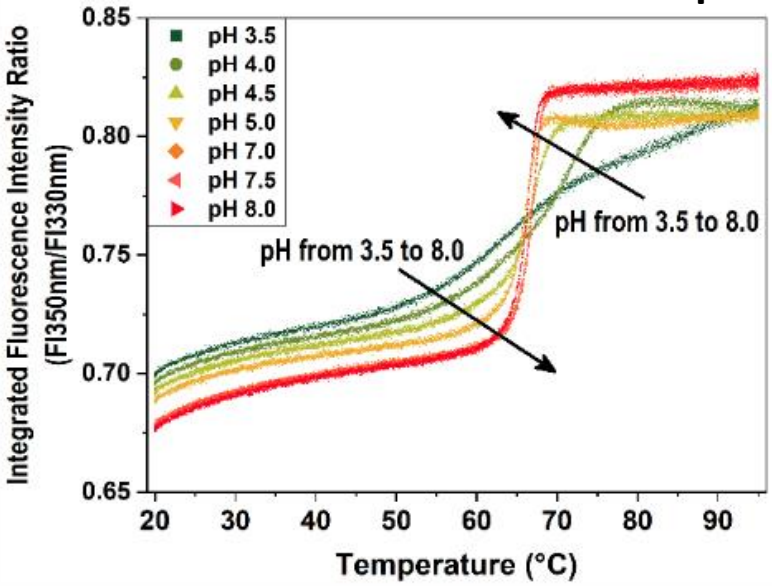


nanoDSF provides melting temperatures like other methods faster and with less sample

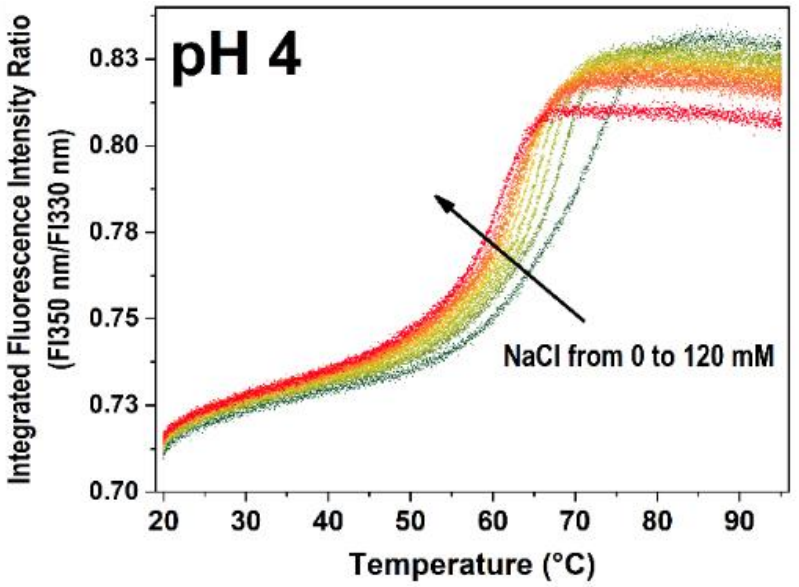
µDSC	near-UV CD	nanoDSF
≈ 550 µL	≈ 600 µL	≈ 10 µL
≈ 3 samples/day	≈ 6 samples/1.5 h	≈ 48 samples/1.5 h
Extensive hands-on time	Medium hands-on time	Very short hands-on time



nanoDSF: Thermal unfolding of interferon α2a at different pH



nanoDSF: Thermal unfolding of interferon α2a as a function of [NaCl]



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The data on these graphs is generated in 90 minutes with 150 µL of sample

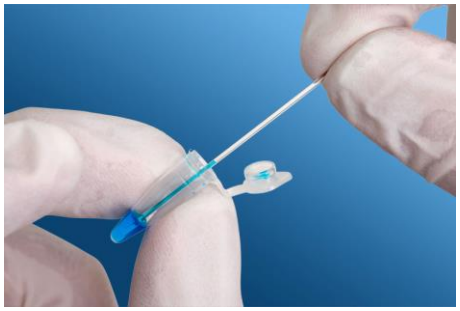
Rapid sample-saving biophysical characterisation and long-term storage stability of liquid interferon alpha2a formulations: Is there a correlation?

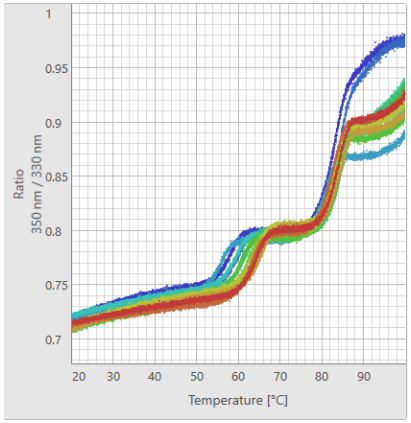
Hristo Svilenov*, Gerhard Winter

Department of Pharmacy, Pharmaceutical Technology and Biopharmaceutics, Ludwig-Maximilians-University, Butenandtstrasse 5-13, Munich D-81377, Germany

we investigate the effect of pH and ionic strength on protein stability. The predictions from the sample-saving biophysical characterisation are validated by long-term stability studies at 4 °C and 25 °C for 12 months on selected formulations. Interferon alpha2a shows minimal aggregation in 10 mM sodium acetate buffer with pH 4 and low ionic strength. The latter is indicated by the rapid sample-saving biophysical characterisation and confirmed by the long-term stability data.

The T_m approach with nanoDSF in brief





Measuring T_m with nanoDSF offers:

- Some predictions which formulations are stable during storage
- High-throughput: typically 48 samples in 1.5 hours
- Small sample volume: 10 µL for one measurement

However, this approach has the following limitations:

- Sample heating: some excipients change properties with temperature
- Limited prediction reliability: T_m shows a limitation in predicting the exact ranking

Thermal denaturation techniques like DSC and nanoDSF work by increasing sample temperature to measure melting temperatures

molecular pharmaceutics

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Article

Cite This: *Mol. Pharmaceutics* 2018, 15, 256−267

T_m-Values and Unfolded Fraction Can Predict Aggregation Rates for Granulocyte Colony Stimulating Factor Variant Formulations but Not under Predominantly Native Conditions

Mathew J. Robinson,[†] Paul Matejtschuk,[§] Adrian F. Bristow,[§] and Paul A. Dalby*[,†]

[†]Department of Biochemical Engineering, University College London, London WC1E 7JE, U.K.

[§]National Institute of Biological Standards and Control (NIBSC), South Mimms, Potters Bar, Hertfordshire EN6 3QG, U.K.

Overall, as a formulation development strategy, it is useful to increase T_m in the initial stages for poorly stable proteins, at least up until the mechanistic limit is reached whereby $f_{T37} < 10^{-3}$. For degradation of GCSF at 37 °C, this limit was reached at approximately $T_m > 55$ °C, where $\ln \nu_{mon} < 4$ (% day^{-1}). Beyond that point, a very different formulation strategy would be required. Protein engineering to remove aggregation hotspots, minimize local unfolding dynamics, increase the net charge, or remove hydrophobic surface patches could be considered.

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ELSEVIER

Research paper

Isothermal chemical denaturation as a complementary tool to overcome limitations of thermal differential scanning fluorimetry in predicting physical stability of protein formulations

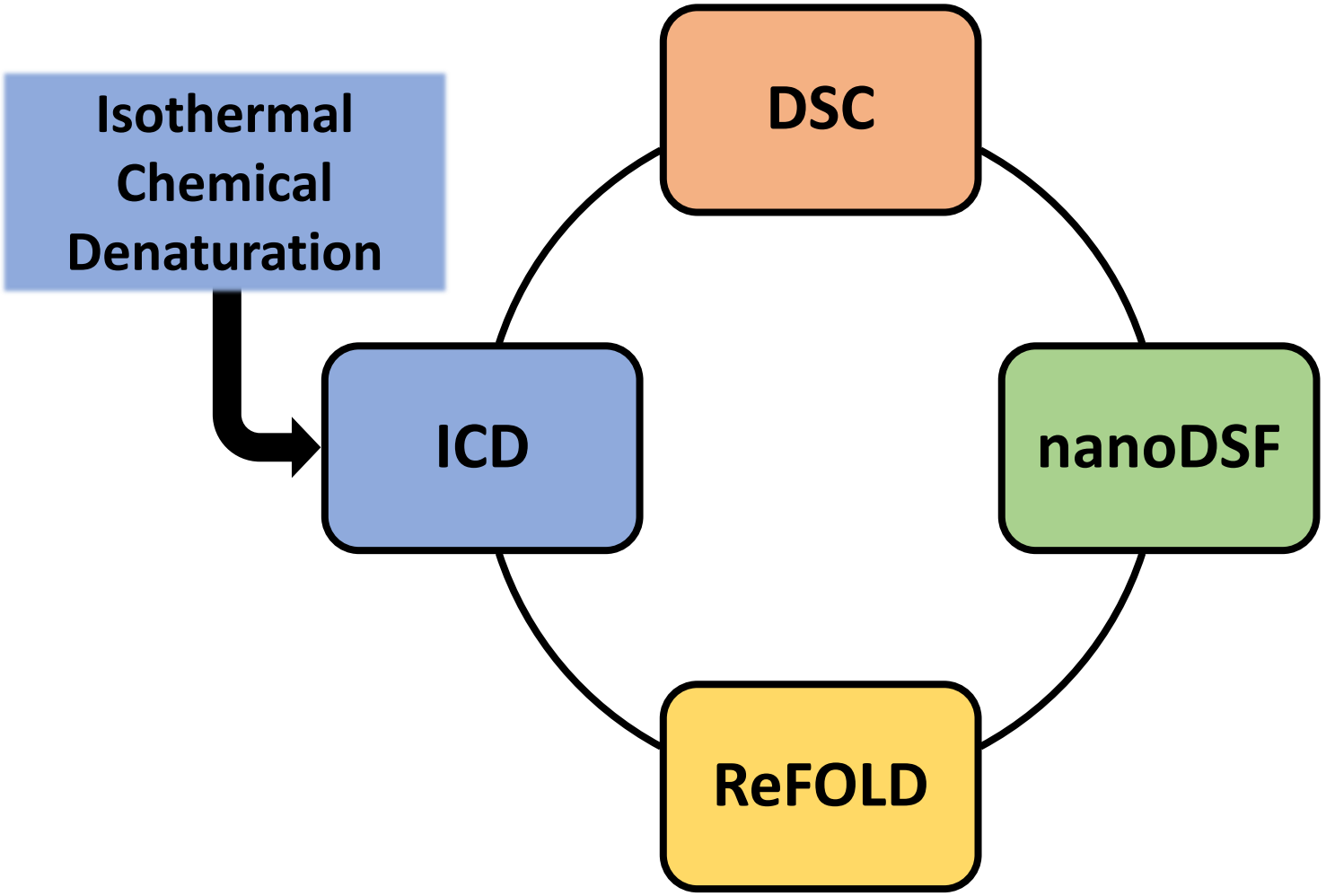
Hristo Svilenov[a,*], Uroš Markoja[b], Gerhard Winter[a]

[a] Department of Pharmacy, Pharmaceutical Technology and Biopharmaceutics, Ludwig-Maximilians-Universität München, Butenandtstrasse 5-13, Munich D-81377, Germany

[b] University of Ljubljana, Faculty of Pharmacy, Aškerčeva 7, 1000 Ljubljana, Slovenia

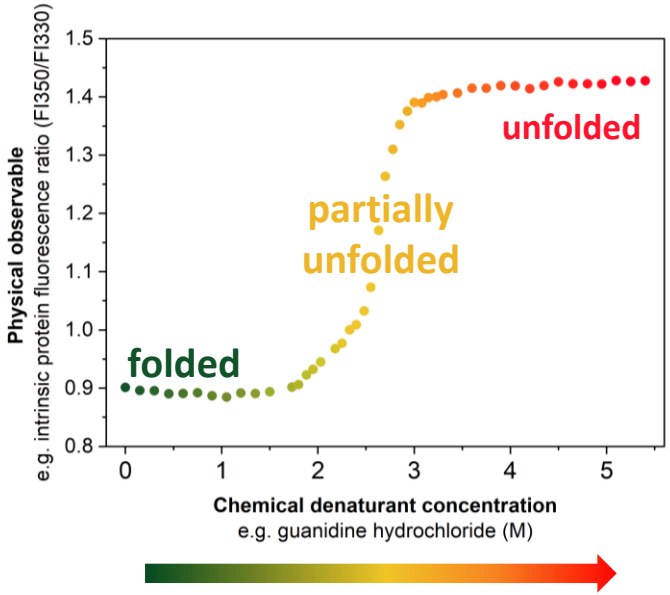
High throughput thermal denaturation is a valuable technique to determine the melting temperatures of therapeutic protein candidates in early stage development when the amount of material is limited. When it comes to formulation studies, thermal denaturation techniques in general are (alongside other pitfalls discussed in the introduction) limited by the fact that the increase in temperature can change key properties of the excipients (i.e. pH of the buffer system). Care should be taken when such measurements are conducted. pH screenings based on T_m values should be performed only in buffers with dpH/dT close to zero. After the pH range of maximum thermal stability of a protein is

➢ **High demand for orthogonal isothermal techniques**

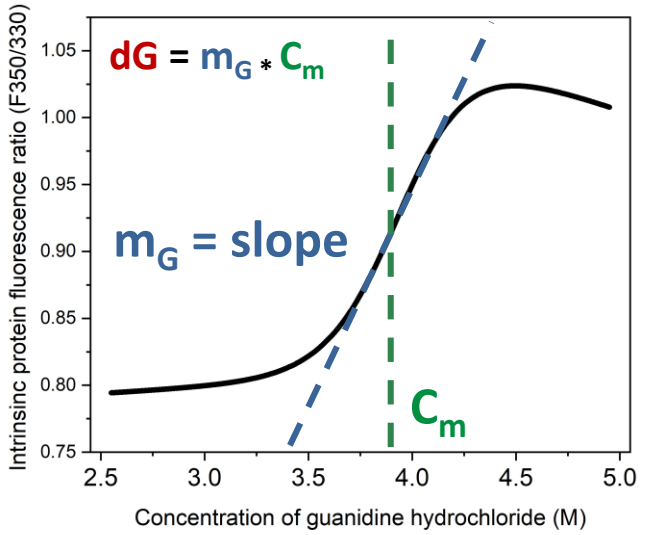

Isothermal Chemical Denaturation
DSC
ICD
nanoDSF
ReFOLD

Isothermal Chemical Denaturation (ICD) for protein formulation

Increasing concentrations of a chemical denaturant are used to cause protein unfolding



Fit to a two-state protein unfolding in an ICD experiment



The ICD data can provide the following protein stability-indicating parameters:

C_m – the "melting" concentration of denaturant needed to unfold 50 % of the protein;

m_G – the "m-value" is an indicator for the cooperativity of the unfolding;

dG – the Gibbs free energy of protein unfolding;

Freire et al. (2013) *Drug discovery today*, 18(19-20), pp. 1007-1013.

Pace (1986) In *Methods in enzymology* (Vol. 131, pp. 266-280). Academic Press.

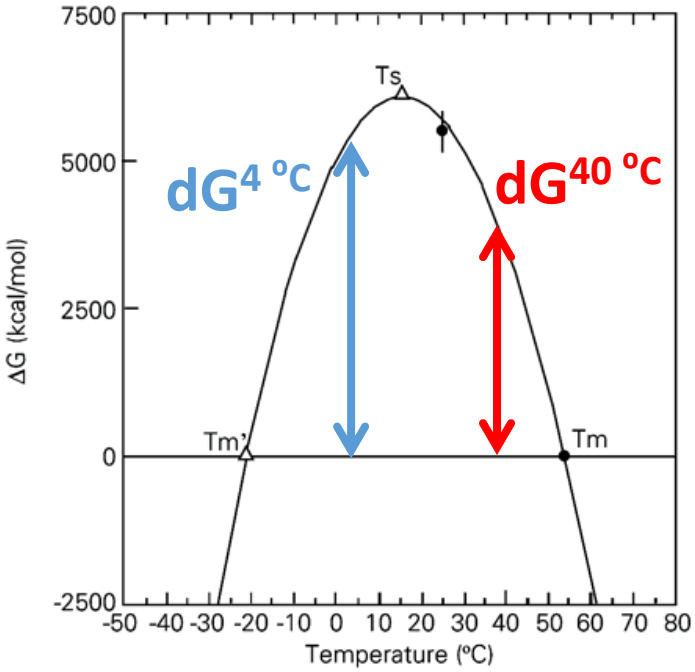
The true **dG allows to calculate the fraction of unfolded protein species**

$$\text{Unfolded} \underset{}{\overset{K_{eq}}{\rightleftharpoons}} \text{Folded}$$

$$dG = -RT\ln(K_{eq})$$

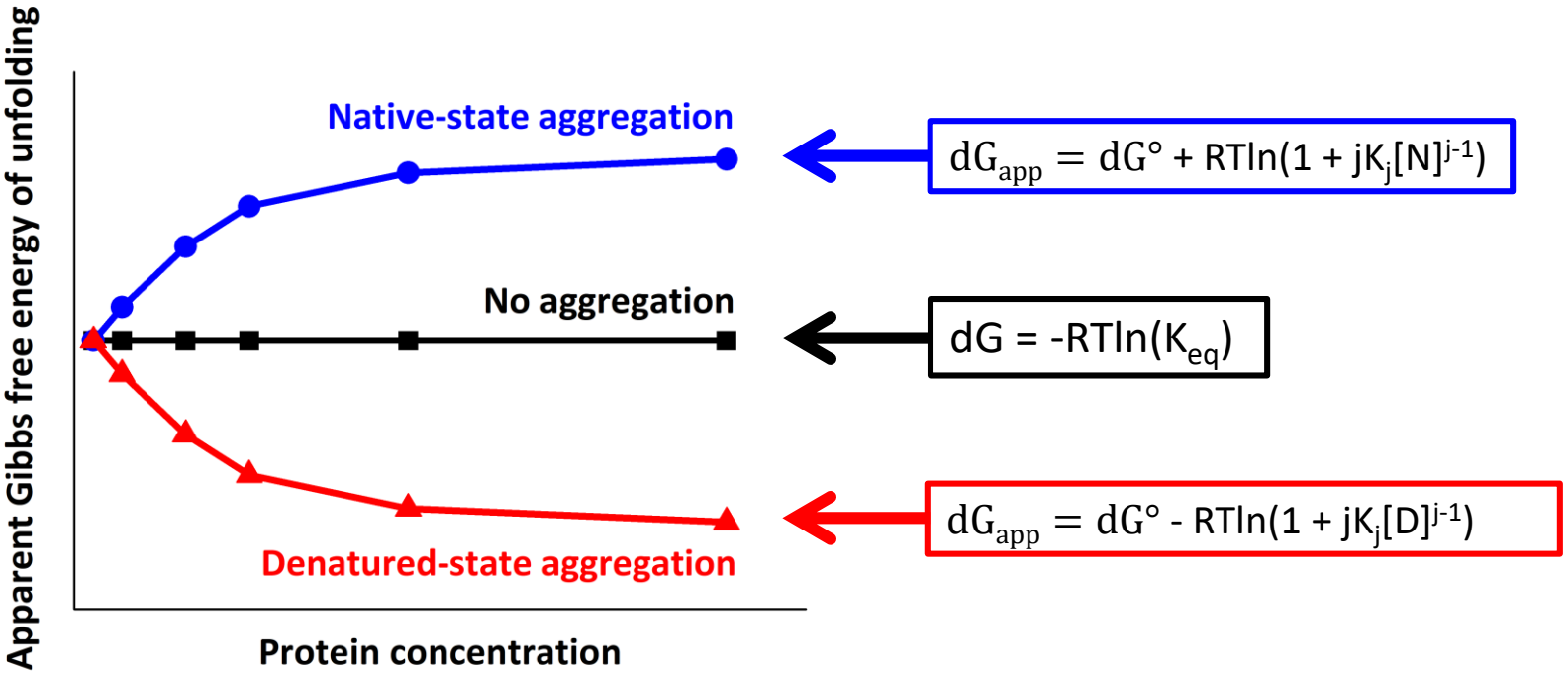
dG (kcal/mol)	% Unfolded protein
0	50
2.7	1
4.1	0.1
5.5	0.01
6.8	0.001
8.2	0.0001
9.6	0.00001

dG is a measure of the protein conformational stability at a given temperature.



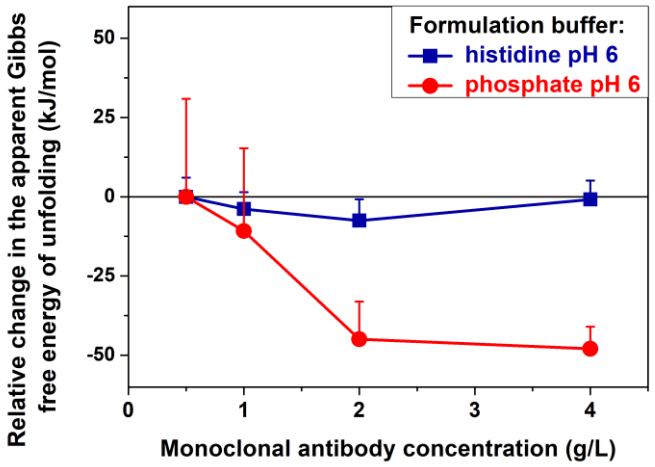
Determining the true dG is possible only when the unfolding of the protein in the denaturant is fully reversible

The concentration dependence of the apparent dG can indicate whether a protein is likely to aggregate from its native or unfolded state

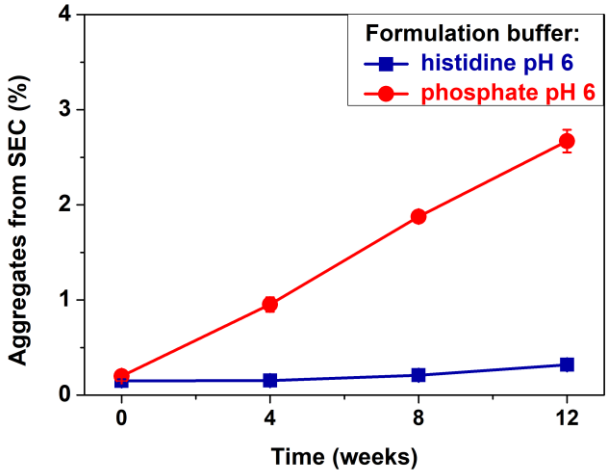


Schön et al. (2015) *Analytical biochemistry*, 488, pp. 45-50.; Clarkson et al. (2016) *Drug discovery today,* 21(2), pp. 342-347.;

Concentration dependence of the Gibbs free energy (dG) of protein unfolding



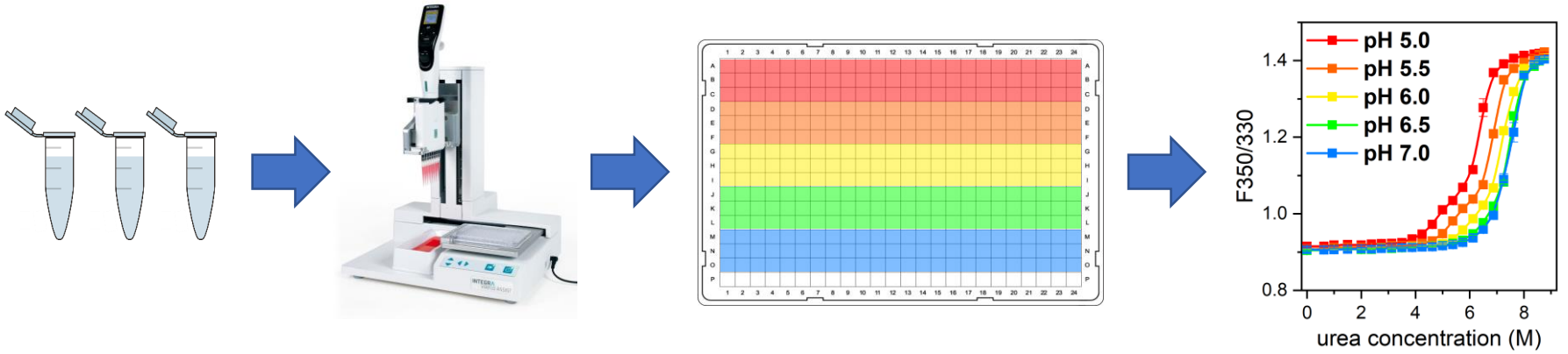
Protein aggregation at 40 °C



This approach is useful and provides orthogonal information during the selection of stable protein formulations

Svilenov, H., Markoja, U. and Winter, G., 2018.. *Eur J Pharm Biopharm*, 125, pp.106-113.

The dG and dG_{trend} approach with ICD in brief

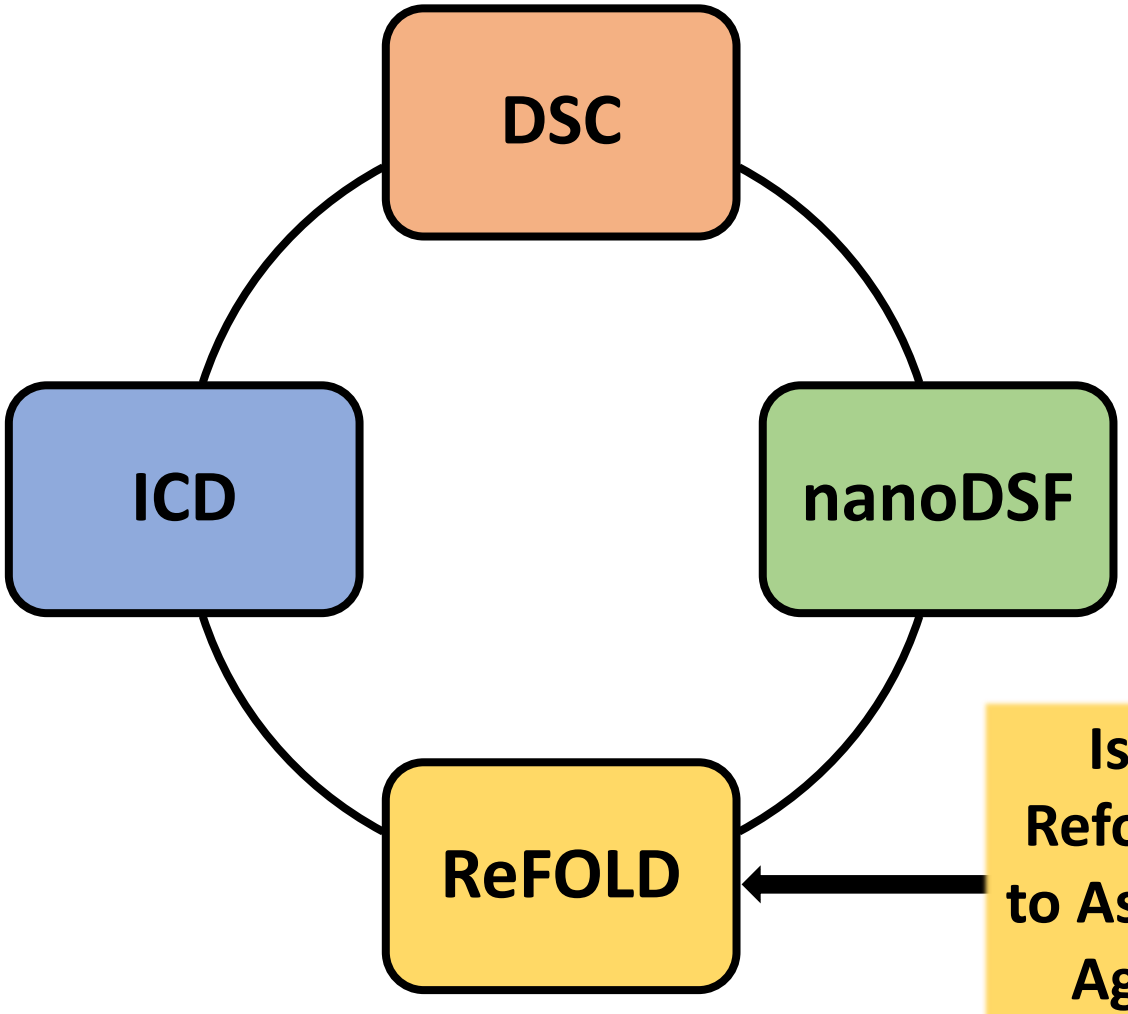


Measuring dG and the concentration dependence of dG with ICD offers:

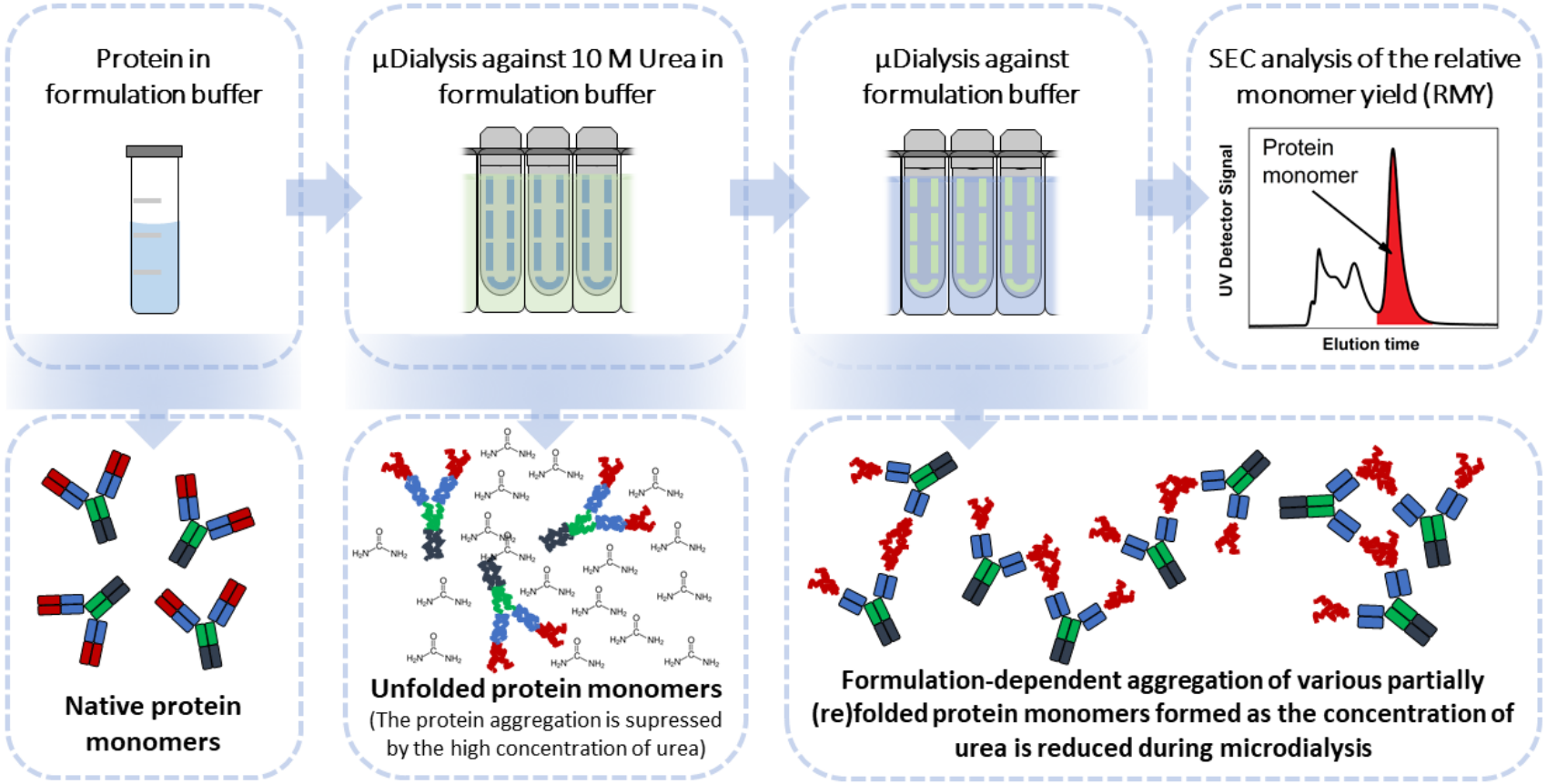
- Some predictions which formulations are stable during storage
- No sample heating

However, this approach had the following limitations:

- Medium throughput: Depends on the experimental set-up
- Medium to high sample volume: Depends on the experimental set-up
- Limited prediction accuracy: The exact order of the formulations is still not known


DSC
nanoDSF
ReFOLD
ICD
Isothermal Refolding Assay to Assess Protein Aggregation

Schematic diagram of the ReFOLD assay



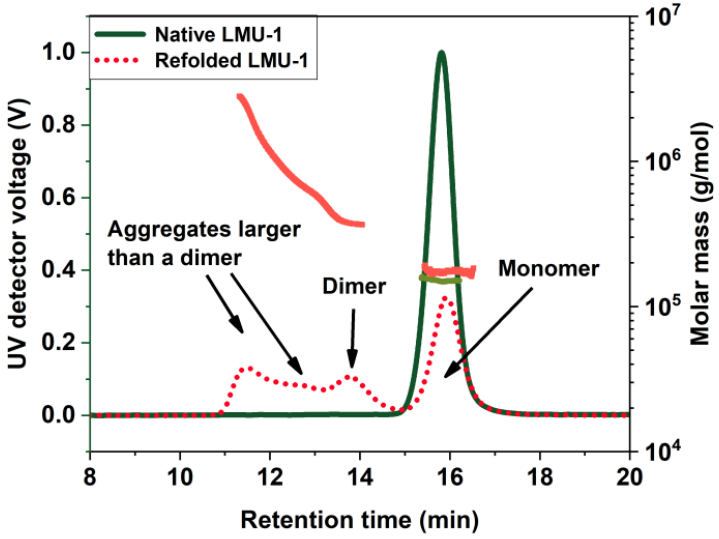
Basic assumption of this approach:

The aggregation of the partially unfolded protein during refolding correlates with the long-term storage stability

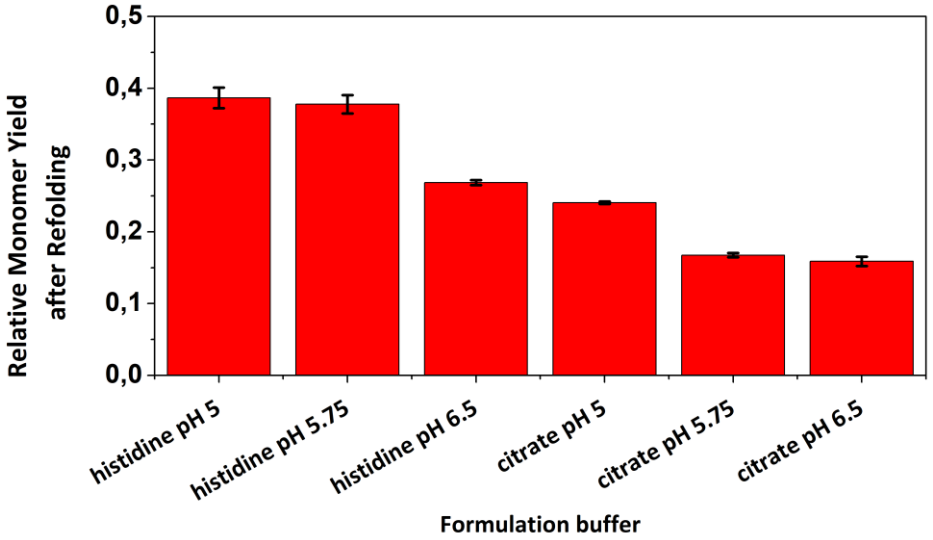
Svilenov, H. and Winter, G., 2019.. *Eur J Pharm Biopharm,* 137, pp. 131-139

Protein aggregation during dialysis refolding depends on the formulation

SEC-MALS chromatograms of native and refolded mAb-A



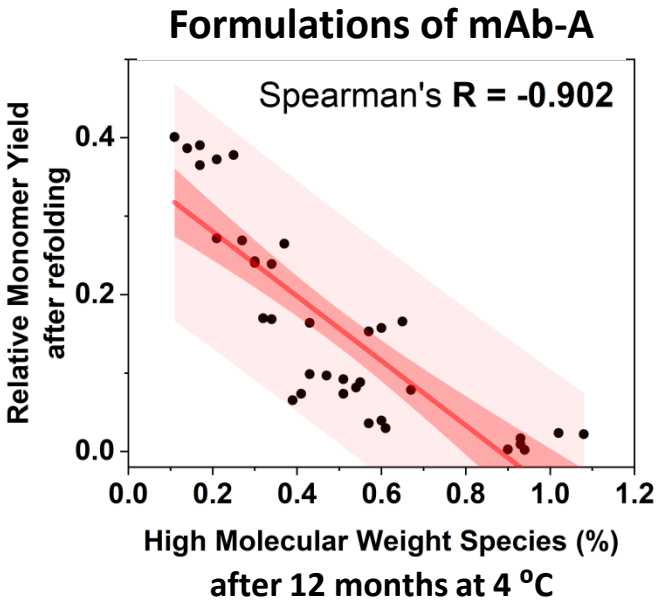
Effect of formulation on the relative monomer yield of mAb-A after refolding from urea

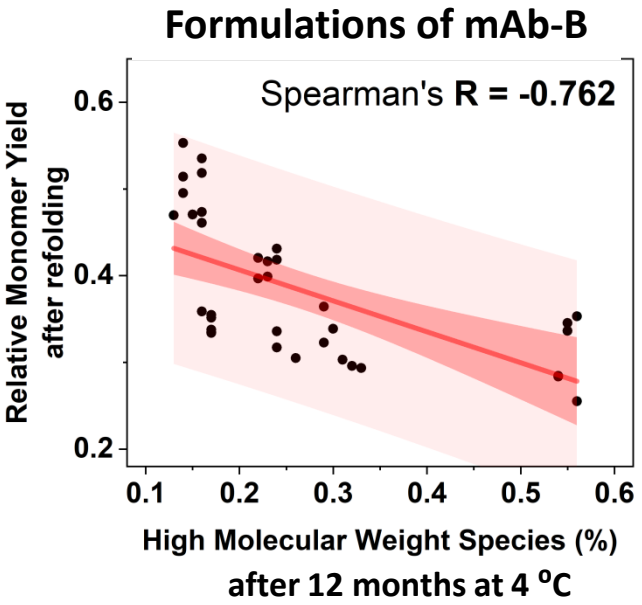


? Can this approach rank the storage stability of the formulations? ?

Svilenov, H. and Winter, G., 2019.. *Eur J Pharm Biopharm,* 137, pp. 131-139

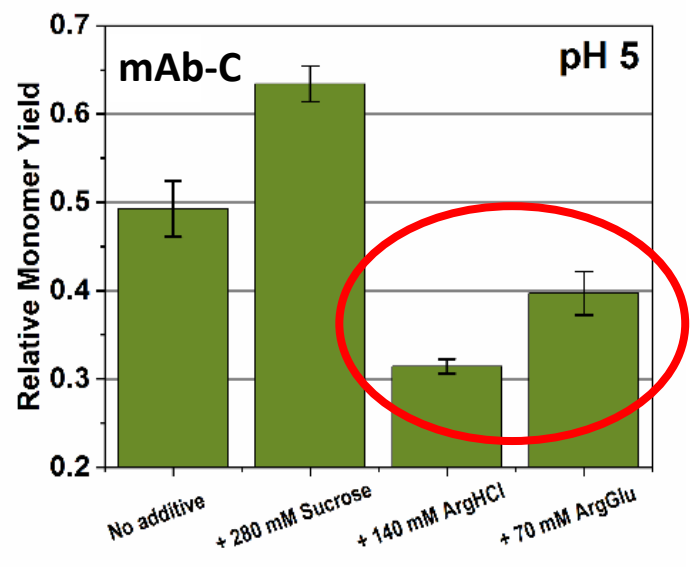
The relative monomer yield from the ReFOLD assay correlates well with the amount of protein aggregates detected after long-term storage for 12 months at 4 °C

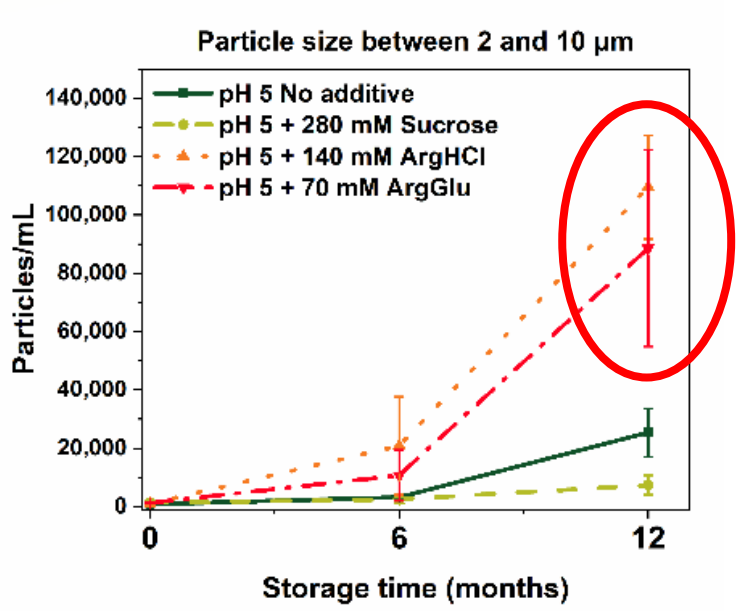




Svilenov, H. and Winter, G., 2019.. *Eur J Pharm Biopharm,* 137, pp. 131-139

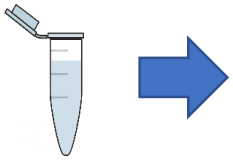
The relative monomer yield from the ReFOLD assay correlates well with the number of subvisible particles detected after storage for 12 months at 25 °C

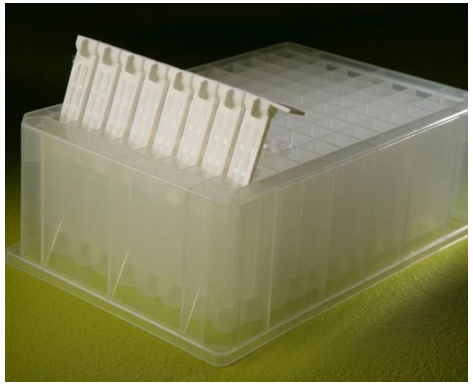


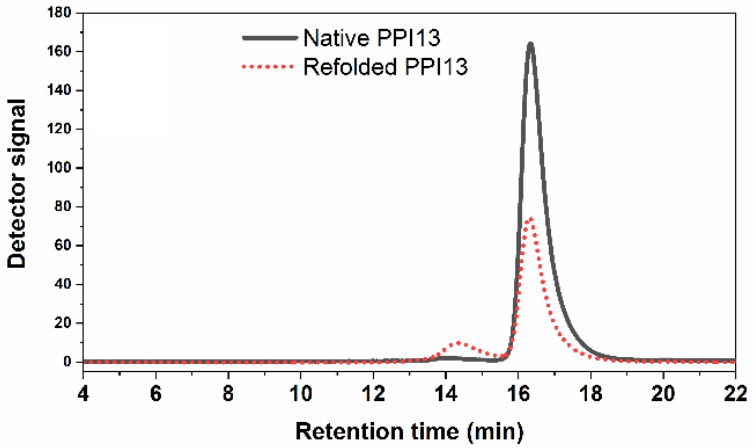


Svilenov, H. et al. *In preparation*

The approach with the ReFOLD assay in brief



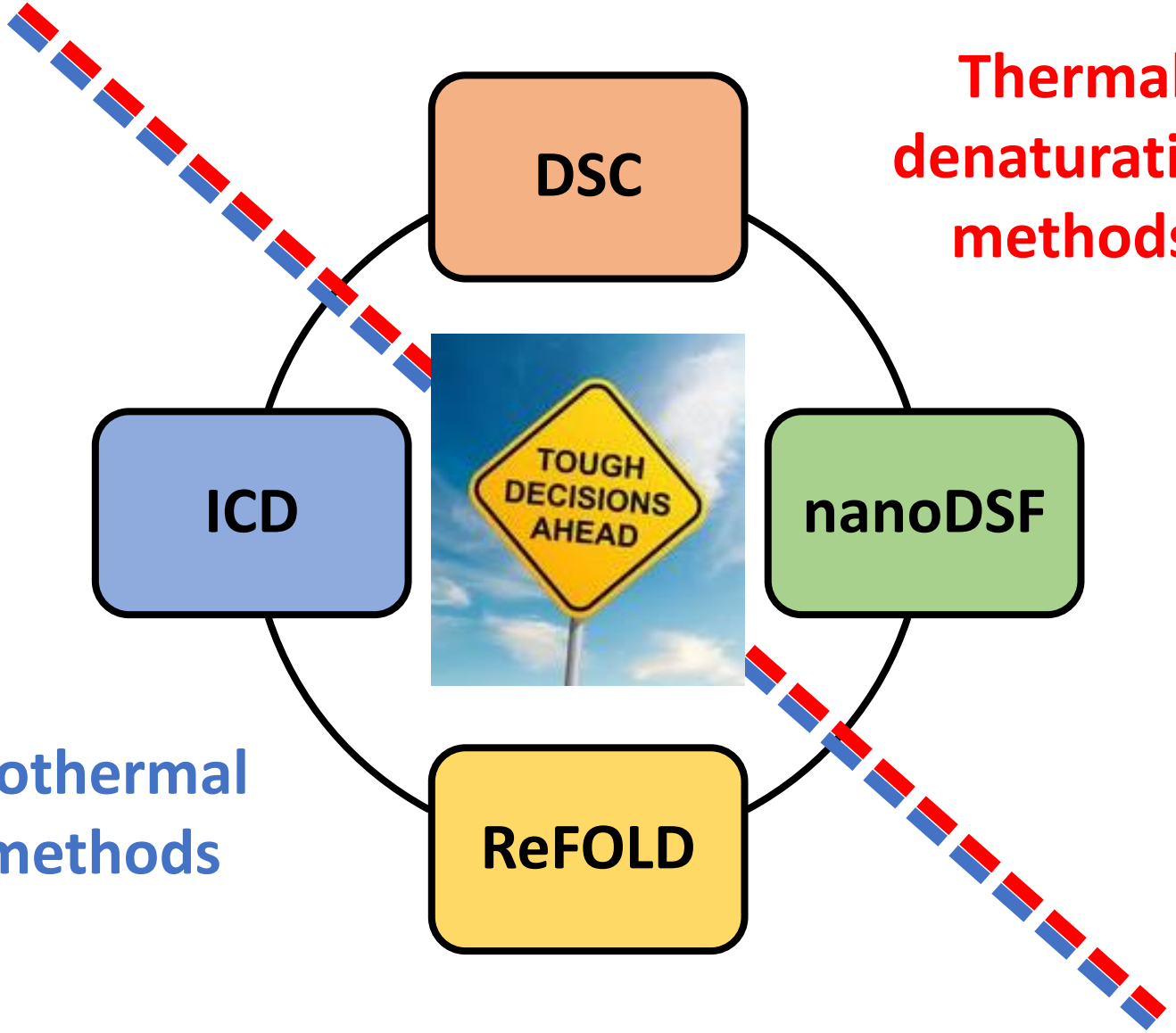




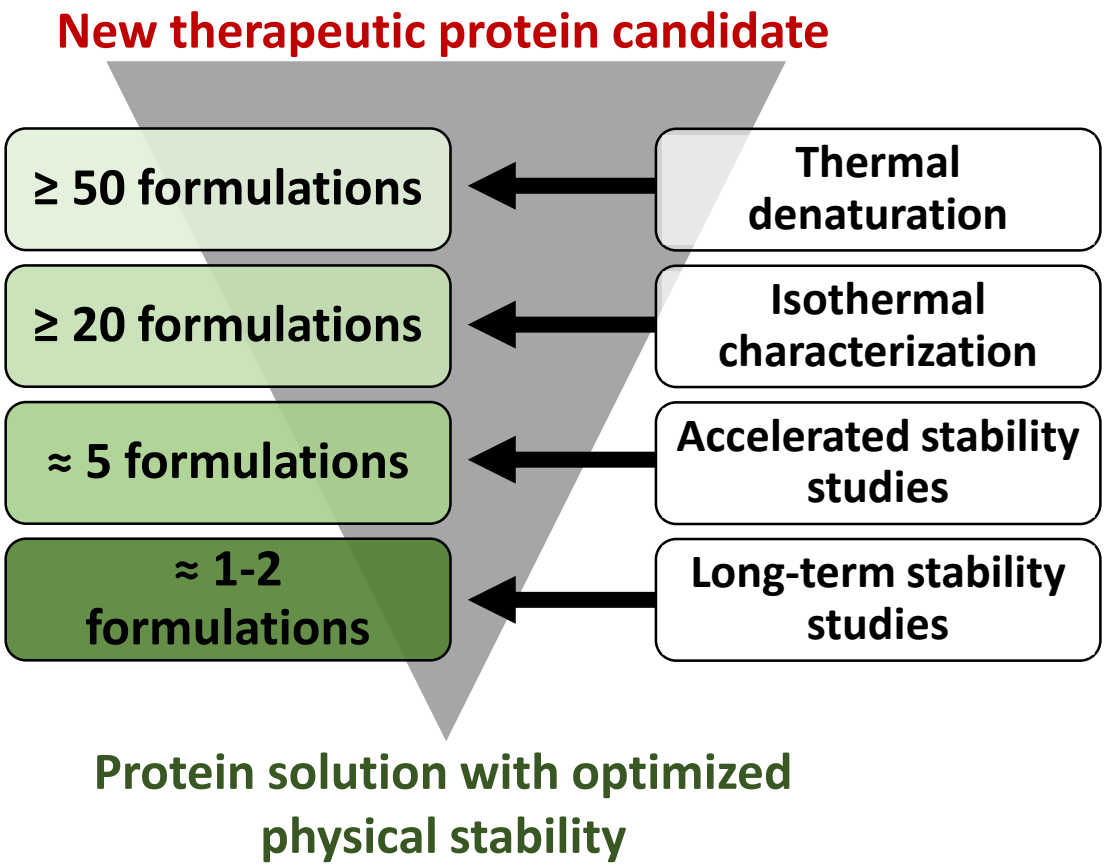
The ReFOLD assay offers:

- Good predictions which formulations are stable during storage
- No sample heating
- Medium to high throughput: 96 well plate, <48 hours to complete
- Small to medium sample volume: between 10 and 100 µL

Which stability-indicating method to choose?



How different methods can be combined in formulation development



nanoDSF (DSC as backup)
Low volume, Fast, Useful, Limitations

ReFOLD (also ICD, DLS, SLS)
Low volume, Better predictions

Storage at 25 °C

Storage at 4 °C

In summary:

- **Use a combination of stability-indicating methods**
- **Know the strengths and limitations of each method**
- **Validate novel predictive methods with long-term stability data**

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MANCHESTER 1824 The University of Manchester

Dr. Chris van der Walle

Dr. Philipp Baaske
Dr. Nuska Tschammer

Dr. Dierk Roessner
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Thank you for your attention!